

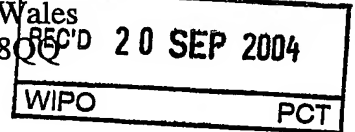


GB04/03341



INVESTOR IN PEOPLE

The Patent Office  
 Concept House  
 Cardiff Road  
 Newport  
 South Wales  
 NP10 8QQ



# **PRIORITY DOCUMENT**

SUBMITTED OR TRANSMITTED IN  
 COMPLIANCE WITH RULE 17.1(a) OR (b)

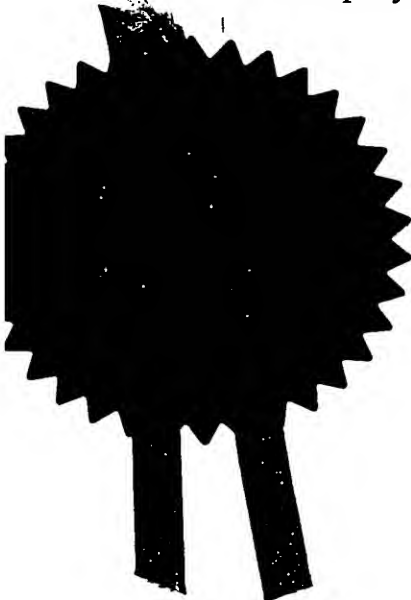
I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., ple, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

BEST AVAILABLE COPY



*P. Mahoney*

Signed

Dated 17 August 2004



30 JUL 03 EB26300-1 D10180  
P01/7700-0100-0317743.3

# Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

30 JUL 2003

The Patent Office

Cardiff Road  
Newport  
South Wales  
NP10 8QQ

1. Your reference PA0356-GB

2. Patent application number  
(The Patent Office will fill in this part)

0317743.3

3. Full name, address and postcode of the or of each applicant (underline all surnames)

AMERSHAM BIOSCIENCES UK LIMITED  
Amersham Place  
Little Chalfont  
Buckinghamshire  
HP7 9NA

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

08331985001  
United Kingdom

4. Title of the invention

METHOD FOR MEASURING AROMATASE ACTIVITY

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

HAMMER, Catriona, MacLeod ; ROLLINS, Anthony, John;  
HAMMETT, Audrey, Grace, Campbell and BRYAN, Ian, Bennett  
Amersham plc  
Amersham Place  
Little Chalfont  
Buckinghamshire HP7 9NA

Patents ADP number (if you know it)

08331985001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (if you know it) the or each application number

Country

Priority application number  
(if you know it)

Date of filing  
(day / month / year)

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing  
(day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (Answer 'Yes' if:

Yes

- a) any applicant named in part 3 is not an inventor, or
  - b) there is an inventor who is not named as an applicant, or
  - c) any named applicant is a corporate body.
- See note (d))

**Patents Form 1/77**

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form

Description 27 ✓

Claim(s) 7 ✓

Abstract 1 ✓

Drawing(s) 5 + 5 ✓

10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*) 1 ✓

Request for substantive examination (*Patents Form 10/77*)

Any other documents  
(please specify)

11.

I/We request the grant of a patent on the basis of this application.

BRYAN, Ian, Bennett

Signature

Ian B. Bryan

Date

29 July 2003

12. Name and daytime telephone number of person to contact in the United Kingdom BRYAN, Ian, Bennett 01494 542090

**Warning**

*After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.*

**Notes**

- a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.  
b) Write your answers in capital letters using black ink or you may type them.  
c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.  
d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.  
e) Once you have filled in the form you must remember to sign and date it.  
f) For details of the fee and ways to pay please contact the Patent Office.

# Method for Measuring Aromatase Activity

## Technical Field

- 5 The present invention relates to compounds, methods and a kit for measuring aromatase activity. The invention can be used for determining both *in vitro* and *in vivo* enzyme activity and for identifying/characterising inhibitors.

## Background to the Invention

10

Assays for measuring enzyme activity are widely employed in the pharmaceutical and environmental sciences. With the advent of combinatorial chemistry and high throughput screening there is a growing need for simple, sensitive and cost-effective assays to screen for potential modulators of enzyme activity.

15

The enzyme aromatase cytochrome P450 19 A1 (EC 1.14.14.1) is the product of the CYP19 gene, a member of the P450 superfamily of genes. Aromatase catalyses the rate-limiting step in oestrogen biosynthesis, the conversion of C<sub>19</sub> androgenic steroids to the corresponding oestrogen (Figure 1), a reaction termed aromatisation since it converts the  $\Delta^4$ -3-one ring of the androgen to the phenolic A-ring of oestrogen (Ciolino *et al.*, 2000, *British Journal of Cancer*, 83, 333-337).

20

- 25 Oestrogens are the most important etiological factors in the growth and development of many breast carcinomas in both pre- and post-menopausal women. Breast tumours from post-menopausal women contain high levels of 17 $\beta$ -oestradiol despite the presence of low plasma 17 $\beta$ -oestradiol concentrations. It is now widely accepted that breast tumours can synthesise 17 $\beta$ -oestradiol from adrenal androgen precursors. Synthesis occurs through the aromitisation of androstenedione to oestrone by aromatase, followed by conversion of oestrone to 17 $\beta$ -oestradiol by 17 $\beta$ -hydroxysteroid dehydrogenase type 1 (James *et al.*, 2001, *Endocrinology* 142, 1497-1505).

30

When measured *in-vitro*, aromatase activity was found to be higher in breast tumours than in adjacent or healthy fat cells. Furthermore, adipose stromal cells surrounding cancerous cells have been shown to contain higher levels of aromatase mRNA than corresponding cells in non-cancerous areas (Chen *et al.*, 1999, *Endocrine-Related Cancer*, 6, 149-156). Thus aromatase activity in tumours or surrounding tissue is believed to play a significant role in promoting tumour growth due to local production of oestrogen.

Aromatase offers a key point of intervention in the treatment of breast cancer by reducing the activity and consequently the level of oestrogen synthesised at the site of the tumour. Thus aromatase inhibitors provide significant benefit to many breast cancer patients (James *et al.*, 2001, *Endocrinology* 142, 1497-1505).

Aromatase is an important enzyme not only from a medical and pharmaceutical viewpoint in the treatment of breast cancer but also from an environmental perspective because inhibitors have been identified as potential environmental toxins, or so called 'endocrine disrupters' (Mak *et al.*, 1999, *Environmental Health Perspectives*, 107, 855-860). The development of a simple, high throughput screening assay to identify modulators and particularly inhibitors of aromatase activity is thus of considerable commercial interest.

#### Fluorescence Detection Methods

Fluorescence-based assays offer significant advantages over radiochemical, ELISA, antibody and more traditional techniques for measuring enzyme activity in terms of simplicity of handling, sensitivity, cost and ease of automation. Recently there has been considerable interest in the application of fluorescence resonance energy transfer (FRET) assays which involve the use of substrates having donor and quenching acceptors on the same molecule. WO 94/28166, for example, reports the use of such FRET labels attached to a polypeptide substrate which fluoresce more intensely on hydrolysis by a protease.

While FRET techniques offer greater sensitivity and reliability for use in screening assays than simple fluorescent intensity techniques, the substrates are considerably more expensive to prepare and purify due to their complex nature. Thus the preparation of FRET labels is demanding in terms of both analytical and/or purification and material costs. Furthermore the only method for distinguishing conventional fluorescent or FRET labels is by their absorption and emission spectra.

Fluorescence lifetime measurements that may be utilised in the present invention offer significant advantages over conventional fluorescence techniques that are based solely on quantifying fluorescence intensity. Fluorescence lifetime is determined from the same spectrally resolved intensity signal, but is additionally resolved in the temporal domain.

Fluorescence lifetime techniques provide greater discrimination because the signal is largely unaffected by 'background noise'. A further advantage with this technique is that several different events can be measured simultaneously by selecting labels having distinguishable lifetimes, thus enabling multiplexing. In addition, measurements of fluorescence lifetime are unaffected by concentration effects and photobleaching.

### Aromatase Assays

Several assay formats have been reported for the measurement of aromatase activity. These can be divided into two categories depending on the use of a 'natural' or a surrogate substrate. Detection methodologies have included the use of radioisotopic tracers (e.g. Thompson & Siiteri, 1974, *Journal of Biological Chemistry*, 249, 5364-5372), fluorescence intensity (Crespi *et al.*, *Analytical Biochemistry*, 1997, 248, 188-190), enzyme activity (e.g. Chabab *et al.*, 1986, *Journal of Steroid Biochemistry*, 25, 165-169) and fast liquid chromatography (Fauss & Pyerin, 1993, *Analytical Biochemistry*, 210, 421-423).

Odum and Ashby (*Toxicology Letters* (2002), 129, 119-122) describe a radiometric assay for measuring aromatase activity using the 'tritiated water assay'. The assay quantifies enzyme activity based on the release of  $^3\text{H}$  as  $^3\text{H}_2\text{O}$  from the  $1\beta$  position of the substrate during aromatisation. A final  
5 reaction contained rat ovary microsomes and an NADPH generating system together with the substrate  $1\beta(^3\text{H})$ -androstenedione and potential aromatase inhibitors in dimethyl sulphoxide. Reactions were started by addition of the substrate and were carried out at  $37^\circ\text{C}$  for 30 min. Reactions were stopped by addition of chloroform-methanol and the mixture shaken for 60 s. After  
10 removal of the solvent, a suspension of dextran-coated charcoal was added. The mixture was left for 1 h at  $4^\circ\text{C}$ , centrifuged and  $500\mu\text{l}$  of the supernatant added to scintillant and counted in a liquid scintillation counter.

Although this assay has been widely used in the literature (e.g. WO  
15 03/045925) as a means for identifying potential inhibitors it is clearly not amenable to high throughput procedures as it is a labour intensive and time-consuming, requiring radiolabelled substrate.

Crespi *et al.* (*Analytical Biochemistry* (1997), 248, 188-190) describe a  
20 microtitre plate-based fluorimetric intensity assay that can be used to measure the activity of recombinant human aromatase expressed in insect cells and prepared as microsomes. The assay uses dibenzylfluorescein (DBF) as the substrate and reports a number of  $\text{IC}_{50}$  values that are in many cases different from reported values. These differences are reportedly due to  
25 variation in methodology such as substrate choice and the use of cell based systems. The use of a 'surrogate' substrate in this second format may explain why the  $\text{IC}_{50}$  differ from the published values.

There is therefore a continued need in the pharmaceutical and environmental  
30 sciences for improved fluorescence-based assays for measuring aromatase activity. Such assays may have one or more of the following attributes: homogeneity, high sensitivity, good reliability, robustness, simplicity of use, low cost, ease of automation, label specificity and/or more than one form of

detection for distinguishing labelled compounds. Preferably the improved assays display more than one of these features and preferably they display all of these features. The present invention seeks to provide novel reagents and methods for performing such an assay.

5

### Summary of Invention

According to a first aspect of the present invention, there is provided a compound of Formula I:

10

$$R-L-S$$
$$(I)$$

15

wherein

R is a fluorescent dye molecule;

L is an optional linkage group containing one or more atoms comprising hydrocarbon chains which may also contain other atoms such as N, O and S; and

20

S is a molecule comprising a substrate group of the enzyme aromatase

25

characterised in that the fluorescence signal of said compound changes in respect of fluorescence intensity or fluorescence lifetime when the compound is acted upon by an enzyme with aromatase activity.

30

Suitably, R is selected from the group consisting of fluorescein, rhodamine, coumarin, BODIPY<sup>TM</sup> dye, phenoxazine, cyanine, Alexa<sup>TM</sup> fluors, merocyanine, Cy3B, Cy5, Cy5.5, Cy7, acridone, quinacridone and squarate dyes.

35

A range of fluorescent labels are commercially available which could be used as a fluorescent reporter moiety R in accordance with the present invention.

Examples include, but are not limited to, oxazine (e.g. MR 121, JA 242, JA 243) and rhodamine derivatives (e.g. JA 165, JA 167, JA 169) as described in WO 02/081509. Other examples (as described in WO 02/056670) include,

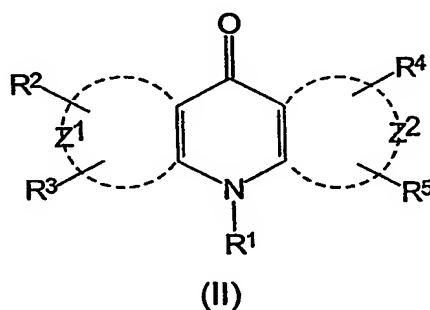
40

but are not limited to Cy5, Cy5.5 and Cy7 (Amersham); merocyanine (Few Chemicals), IRD41 and IRD700 (Licor); NIR-1 and IC5-OSu (Dojindo); Alexa fluor 660 & Alexa fluor 680 (Molecular Probes); LaJolla Blue (Diatron); FAR-Blue, FAR-Green One & FAR-Green Two (Innosense); ADS 790-NS and ADS



821-NS (American Dye Source); indocyanine green (ICG) and its analogues (US Patent No. 5,968,479); indotricarbocyanine (ITC, WO 98/47538); fluorescent quantum dots (zinc sulfide-capped cadmium selenide nanocrystals - QuantumDot Corp.) and chelated lanthanide compounds (fluorescent lanthanide metals include europium and terbium).

Preferably, R is an acridone dye, as described in WO 02/099424, of formula II:



wherein:

groups  $R^2$  and  $R^3$  are attached to the  $Z^1$  ring structure and groups  $R^4$  and  $R^5$  are attached to the  $Z^2$  ring structure;

$Z^1$  and  $Z^2$  independently represent the atoms necessary to complete one or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

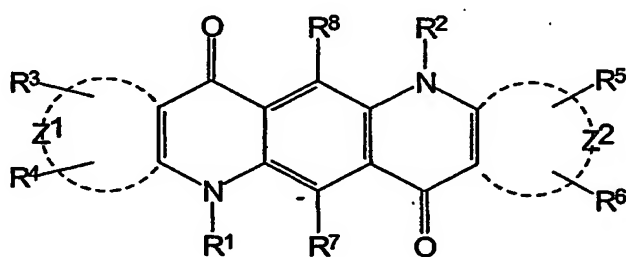
$R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  and  $R^5$  are independently selected from hydrogen, halogen, amide, hydroxyl, cyano, amino, mono- or di- $C_1$ - $C_4$  alkyl-substituted amino, sulphydryl, carbonyl,  $C_1$ - $C_6$  alkoxy, aryl, heteroaryl,  $C_1$ - $C_{20}$  alkyl, aralkyl, the group -E-F where E is a spacer group having a chain from 1-60 atoms selected from the group consisting of carbon, nitrogen, oxygen, sulphur and phosphorus atoms and F is a target bonding group; and the group  $-(CH_2)_nY$  where Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl and n is zero or an integer from 1 to 6.

Suitably, the target bonding group F is a reactive or functional group. A reactive group of the fluorescent dyes according to formula (II) can react under suitable conditions with a functional group of the substrate (i.e. group L or X); a functional group of a compound according to formula (II) can react

under suitable conditions with a reactive group of the substrate. By virtue of these reactive and functional groups, the fluorescent dyes according to formula (II) may be reacted with and covalently bond to the substrate, such that the substrate becomes labelled with the fluorescent dye.

Preferably, when F is a reactive group, it is selected from the group consisting of succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide, haloacetamide, acid halide, vinylsulphone, dichlorotriazine, carbodiimide, hydrazide and phosphoramidite. Preferably, when F is a functional group, it is selected from hydroxy, amino, sulphhydryl, imidazole, carbonyl including aldehyde and ketone, phosphate and thiophosphate.

Preferably, R is a quinacridone dye, as described in WO 02/099432, of Formula III:



(III)

wherein:

groups R<sup>3</sup> and R<sup>4</sup> are attached to the Z<sup>1</sup> ring structure and groups R<sup>5</sup> and R<sup>6</sup> are attached to the Z<sup>2</sup> ring structure;

Z<sup>1</sup> and Z<sup>2</sup> independently represent the atoms necessary to complete one or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

R<sup>1</sup>, R<sup>2</sup>, R<sup>3</sup>, R<sup>4</sup>, R<sup>5</sup>, R<sup>6</sup>, R<sup>7</sup> and R<sup>8</sup> are independently selected from hydrogen, halogen, amide, hydroxyl, cyano, amino, mono- or di-C<sub>1</sub>-C<sub>4</sub> alkyl-substituted amino, sulphhydryl, carbonyl, carboxyl, C<sub>1</sub>-C<sub>6</sub> alkoxy, aryl, heteroaryl, C<sub>1</sub>-C<sub>20</sub> alkyl, aralkyl, the group -E-F where E is a spacer group having a chain from 1-60 atoms selected from the group consisting of carbon, nitrogen, oxygen, sulphur and phosphorus atoms and F is a target bonding group; and the

group  $-(CH_2)_nY$  where Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl and n is zero or an integer from 1 to 6.

- 5 Suitably, the target bonding group F is a reactive or functional group. A reactive group of the fluorescent dyes according to formula (III) can react under suitable conditions with a functional group of the substrate; a functional group of a compound according to formula (III) can react under suitable conditions with a reactive group of the substrate. By virtue of these reactive and functional groups, the fluorescent dyes according to formula (III) may be  
10 reacted with and covalently bond to the substrate, such that the substrate becomes labelled with the fluorescent dye.

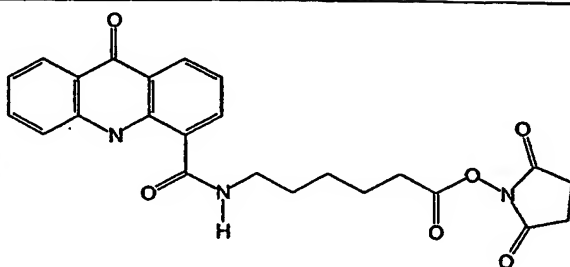
- Preferably, when F is a reactive group, it is selected from the group consisting  
15 of succinimidyl ester, sulpho-succinimidyl ester, isothiocyanate, maleimide, haloacetamide, acid halide, vinylsulphone, dichlorotriazine, carbodiimide, hydrazide and phosphoramidite. Preferably, when F is a functional group, it is selected from hydroxy, amino, sulphydryl, imidazole, carbonyl including aldehyde and ketone, phosphate and thiophosphate.

20

Preferred examples of acridone and quinacridone dyes (and their corresponding lifetimes (nano seconds)) are shown as compounds (IV), (V), (VI), (VII) and (VIII) in Table 1 as their NHS (N-hydroxysuccinimidyl) esters:

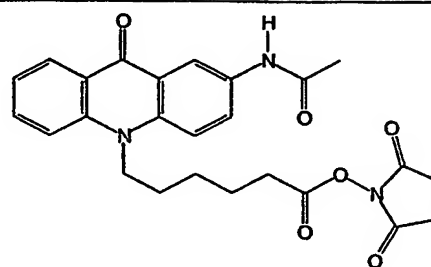
25

Table 1



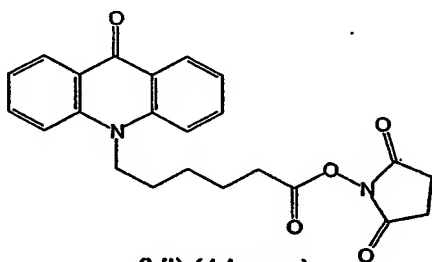
(IV) (4nsec)

O-(N-Succinimidyl)-6-(9-oxo-9H-acridin-4-carboxamido)hexanoate (III)



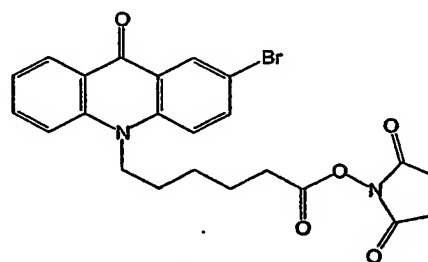
(V) (17nsec)

O-(N-Succinimidyl)-6-(2-acetamido-9-oxo-9H-acridin-10-yl)hexanoate (IV)



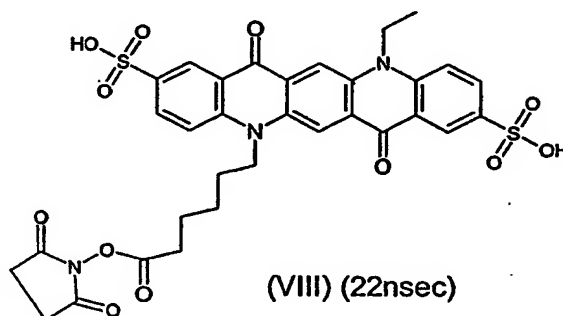
(VI) (14nsec)

O-(N-Succinimidyl)-6-(9-oxo-9H-acridin-10-yl)hexanoate (V)



(VII) (8 nsec)

O-(N-Succinimidyl)-6-(2-bromo-9-oxo-9H-acridin-10-yl)hexanoate (VI)



(VIII) (22nsec)

6-(12-Ethyl-7,14-Dioxo-2,9-disulpho-7,14-dihydroquino[2,3-b]acridin-5(12H)-yl)hexanoic acid succinimidyl ester (VIII)

Suitably, L is a linker group containing from 1 to 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from  $-NR'$ -,  $-O$ -,  $-S$ -,  $-CH=CH$ -,  $-C\equiv C$ -,  $-CONH$ - and phenylenyl groups, wherein  $R'$  is selected from hydrogen and C1 to C4 alkyl.

5

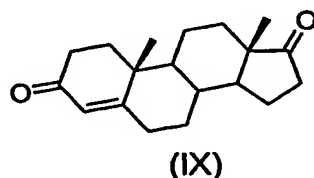
Suitably, L is a linker group containing from 2 to 30 atoms, preferably from 6 to 20 atoms.

Preferably, L is a linker group selected from the group:

- 10  $\{(-CHR')_p-Q-(-CHR')_r\}_s$  where each Q is selected from  $CHR'$ ,  $NR'$ ,  $O$ ,  $-CH=CH-$ ,  $Ar$  and  $-CONH-$ ; each  $R'$  is independently hydrogen or C1 to C4 alkyl; each p is independently 0 to 5; each r is independently 0 to 5; and s is either 1 or 2. More preferably, Q is selected from the group consisting of  $-CHR'$ -,  $-O$ - and  $-CONH-$ , where  $R'$  is hydrogen or C1 to C4 alkyl.

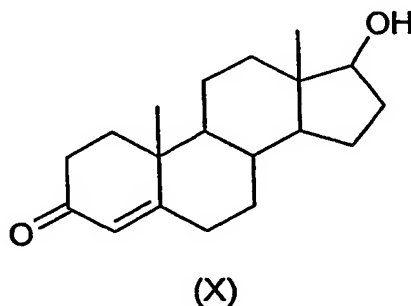
15

Preferably, Group S is androstenedione of Formula IX or a derivative thereof



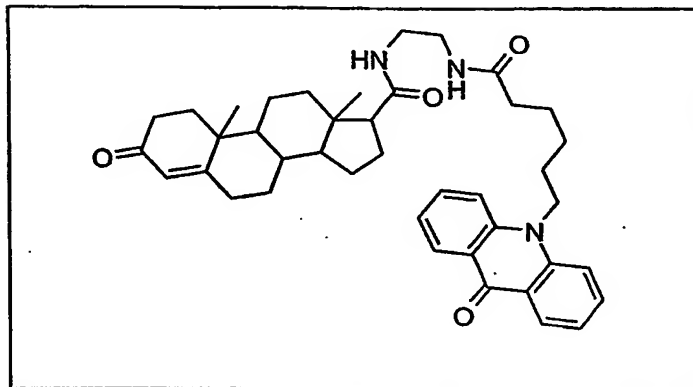
20

Preferably, Group S is testosterone of Formula X or a derivative thereof



25

In a preferred embodiment of the first aspect, there is provided a compound of Formula XI



(XI)

In a second aspect of the present invention, there is provided a method for measuring aromatase activity in a sample, the method comprising the steps of:

- i) measuring the fluorescence intensity or fluorescence lifetime of a compound according to any preceding claim prior to adding it to said sample;
- ii) adding said compound to said sample under conditions which favour aromatase activity, and
- iii) measuring a change in fluorescence intensity or fluorescence lifetime of said compound following step ii);

wherein said change in fluorescence intensity or fluorescence lifetime can be used to determine aromatase activity.

Suitably, the sample is selected from the group consisting of extract, cell, tissue and organism. The cell or organism may be naturally occurring or may be a recombinant cell or organism which has been genetically engineered to over-express a particular protein, such as aromatase.

In a third aspect of the present invention, there is provided a method of screening for a test agent whose effect upon the activity of aromatase is to be determined, said method comprising the steps of:

- 5    i)     performing the method as hereinbefore described in the presence of said agent; and
- ii)    comparing the activity of said aromatase in the presence of the agent with a known value for the activity of aromatase in the absence of the agent;
- 10   wherein a difference between the activity of the aromatase in the presence of the agent and said known value in the absence of the agent is indicative of the effect of the test agent upon the activity of aromatase.

15   A test agent may be, for example, any organic or inorganic compound such as a synthetic molecule or a natural product (e.g. peptide, oligonucleotide), or may be an energy form (e.g. light or heat or other forms of electro magnetic radiation).

20   Suitably, the known value is stored upon an electronic database. Optionally, the value may be normalised (for example, to represent 100% aromatase activity) and compared to the normalised activity of the enzyme in the presence of the test agent. In this way, only test agents affecting enzyme activity by a certain minimum amount may be selected for further evaluation.

25   According to fourth aspect of the present invention, there is provided a method of screening for a test agent whose effect upon the activity of aromatase is to be determined, said method comprising the steps of:

- 30       i)     performing the method of measuring aromatase activity as hereinbefore described in the presence and in the absence of the agent; and
- ii)    determining the activity of said enzyme in the presence and in the absence of the agent;

wherein a difference between the activity of aromatase in the presence and in the absence of the agent is indicative of the effect of the test agent upon the activity of aromatase.

- 5 Suitably, the difference in activity between the activity of the enzyme in the absence and in the presence of the agent is normalised, stored electronically and compared with a value of a reference compound. Thus, for example, the difference in activity may be stored as a percentage inhibition (or percentage stimulation) on an electronic database and this value compared to the
- 10 corresponding value for a standard inhibitor of aromatase. In this way, only test agents meeting a certain pre-determined threshold (e.g. as being as effective or more effective than the reference compound) may be selected as being of interest for further testing.
- 15 The assay method according to the present invention is preferably performed in the wells of a multiwell plate, e.g. a microtitre plate having 24, 96, 384 or higher densities of wells eg. 864 or 1536 wells. Alternatively, the assay may be conducted in assay tubes or in the microchannels of a multifluidic device or in a FACS machine. In a typical assay, a sample containing the substance of
- 20 interest is mixed with the reaction mixture in a well. The reaction is initiated by the addition of enzyme. The reaction is allowed to proceed for a fixed time and stopped with a stop reagent (for example, EDTA).

The reaction mixture can be pre-dispensed into the wells of such a plate.

25

- Typically, enzyme assays are performed under "stopped" conditions. By this it is meant that the reaction is allowed to proceed for a predetermined period and then terminated with a stop reagent. The nature of the stop reagent is typically a strong inhibitor of the enzyme and is often non-specific, for
- 30 example, EDTA, is used to sequester metal ions that are normally present for enzyme activity. In embodiments of the second, third and fourth aspects, assays for aromatase activity either in the presence of or in the absence of a test compound, may be performed under continuous measurement. Because the fluorescence intensity and/or lifetime of the labelled substrate is monitored



continuously and can be seen to change continuously, the labelled substrate does not need separation from the product of the enzymatic reaction. A time-course of the reaction may be obtained in this manner, thus allowing kinetic studies to be performed in real time.

5

In general the assay will consist of several components, typically the enzyme, substrate, cofactors, metal ions, buffer salts and possibly test or standard inhibitor compounds.

- 10 Additionally it may be necessary to run the assays in the presence of low percentages of organic solvents such as DMSO. In this invention it is possible to add any of the reagents to the mix whilst omitting a critical component in any order. This type of reaction can then be monitored for non-specific effects. It is also possible to construct mixture with no enzyme for further controls.
- 15 Due to the nature of the reactions, it is then possible to add the final component and monitor changes either in real time or by stopping the reaction at some point in the future.

- The methods of the invention can be carried out in samples derived from cells, tissues, organisms and extracts. Biological samples may, for example, be
- 20 homogenates, lysates or extracts prepared from whole organisms, parts of an organism or tissues. For example, the assay can be conducted on a variety of body fluids such as blood, mucus, lymphatic fluid, synovial fluid, cerebrospinal fluid, saliva, amniotic fluid, urine, vaginal fluid and semen. In particular, the
- 25 assay may be conducted on adipose or breast tissues and cells.

- Furthermore, it is possible to conduct the assay in media, such as nutrient broth or similar media, where it is possible to grow either eukaryotic or prokaryotic cells. Cells engineered to over-express aromatase, such as JEG3
- 30 choriocarcinoma cells obtained from ATCC (Bhatnager et al., 2001, *Journal of Steroid Biochemistry and Molecular Biology*, 76, 199-202) are particularly useful for screening inhibitors.

Suitably, conventional detection methods can be employed to measure fluorescence intensity and/or the lifetime of the label. These methods include instruments using photo-multiplier tubes as detection devices. Several approaches are possible using these methods; e.g.

5

- i) methods based upon time correlated single photon counting (cf. Principles of Fluorescence Spectroscopy, (Chapter4) ed. J R Lakowicz, Second Edition, 1999, Kluwer/Academic Press)
- ii) methods based upon frequency domain/phase modulation (cf. Principles of Fluorescence Spectroscopy, (Chapter5) ed. J R Lakowicz, Second Edition, 1999, Kluwer/Academic Press)
- iii) methods based upon time gating (cf. Sanders et al., (1995) Analytical Biochemistry, 227 (2), 302-308).

10

Measurement of fluorescent intensity may be performed by means of a charge coupled device (CCD) imager, such as a scanning imager or an area imager, to image all of the wells of a multiwell plate. The LEADseeker<sup>TM</sup> (Amersham Biosciences, UK) system features a CCD camera allowing imaging of high density microtitre plates in a single pass. Imaging is quantitative and rapid, and instrumentation suitable for imaging applications can now simultaneously image the whole of a multiwell plate.

20

According to a fifth aspect of the present invention, there is provided a method for measuring cellular distribution of the compound as hereinbefore described, wherein the compound is capable of being taken up by a living cell, the method comprising the steps of:

25

- i) measuring the fluorescence intensity or the fluorescence lifetime of the compound in a cell-free environment or a parental host cell;
- ii) adding the compound to one or more cells or cells engineered to over-express aromatase, and
- iii) measuring the fluorescence intensity or fluorescence lifetime of the compound following step ii);

30

wherein a change in fluorescence intensity or fluorescence lifetime indicates aromatase activity and can be used to determine the distribution of the

compound in the cell. It will be understood that cells which have been genetically engineered to over-express aromatase compared to their parental host cells will exhibit significantly higher levels of enzyme activity.

- 5 Suitably, the cell is selected from the group consisting of mammalian, plant, insect, fish, avian, bacterial and fungal cells. Cell suspensions are particularly suitable for use in the method of the third aspect of the present invention, although other forms of cell culture may be used which are amenable for cell-based assays.

10

In a sixth aspect of the present invention, there is provided the use of a compound as hereinbefore described for measuring aromatase activity as an *in vitro* or an *in vivo* imaging probe.

- 15 In a seventh aspect of the present invention; there is provided a kit comprising:

- i) a compound as hereinbefore described; and
- ii) an assay buffer; and optionally
- iii) a stop buffer.

20

### Brief Description of the Drawings

Figure 1 illustrates the biochemical activity of aromatase in converting androstenedione to oestrone.

25

Figure 2 shows a comparison of buffer only, control and CYP19 microsomes on fluorescence assay signal.

Figure 3 depicts the effect that microsome volume has on fluorescence assay  
30 signal.

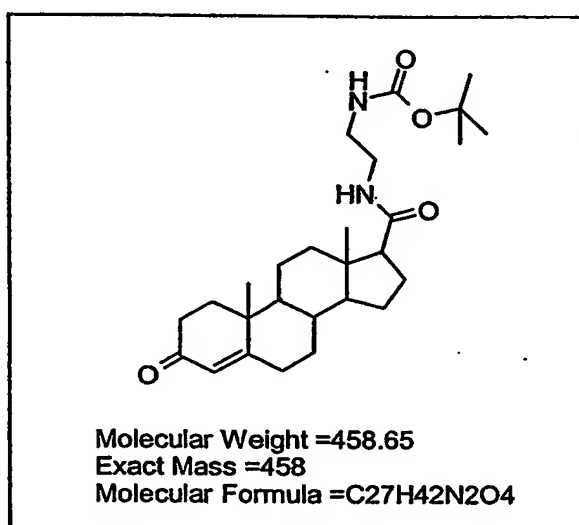
Figure 4 illustrates the NADPH dependence of the microsome preparation (CYP19)/aromatase enzyme activity.

Figure 5 shows the specificity of the aromatase enzyme for its substrate.

### Specific Description

#### Synthesis of Aromatase Substrate

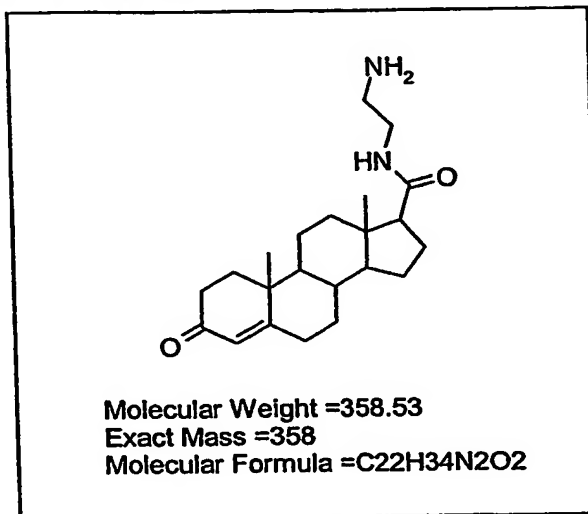
- i) Tert-Butyl 2-[(3-oxoandrost-4-en-17-yl)carbonyl]amino}-  
ethylcarbamate



Formula (XII)

To 0.49g of 4-androsten-3-one-17 $\beta$ -carboxylic acid was added N,N-dimethylformamide (3ml), N,N-diisopropylethylamine (0.55ml) and O-(N-Succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (0.48g). On stirring at room temperature (under an atmosphere of nitrogen) for 1.5 hours tert Butyl N-(2-aminoethyl) carbamate (0.25g) was added. The mixture was stirred at room temperature for 3 days after which time the volatile components were removed on a rotary evaporator. Flash column chromatography was performed and the relevant fractions combined and stripped of solvent using a rotary evaporator. This gave 0.50g of the desired material (Formula XII).

Mass spectrum : 459.30 (M+H)

ii) N-(2-aminoethyl)-3-oxoandrost-4-ene-17-carboxamide

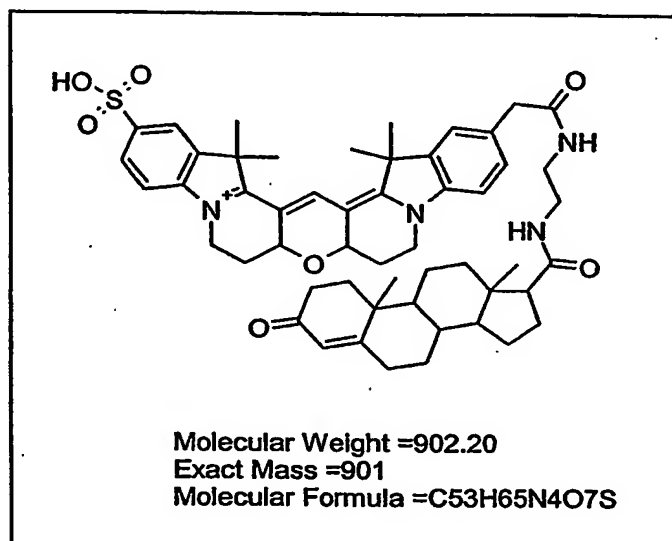
5

Formula (XIII)

To 16.5mg of Tert-Butyl 2-[[[(3-oxoandrost-4-en-17-yl)carbonyl]amino]-ethylcarbamate was added 0.5ml of 95% trifluoroacetic acid/ water. On  
10 standing for 2 hours the volatile components were removed using a rotary evaporator. The resulting product (Formula XIII), which was an oil, was used without further purification. Mass spectrum : 359.23 (M+H)

- iii) N-(2-[(2-(6,7,8,9,10-tetrahydro-14-sulfonato-16,16,18,18-tetramethyl-7aH-bisindolinium[3,2-a,3',2'-a]pyrano[3,2-c,5,6-c]dipyridin-5-ium)acetyl)amino} ethyl)-3-oxoandrost-4-ene-17-carboxamide.

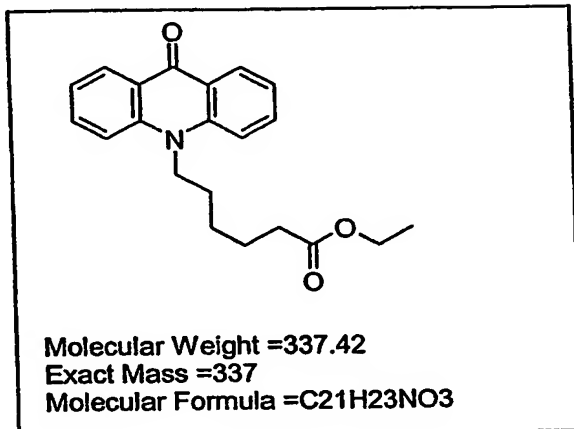
5



Formula (XIV)

To 6,7,8,9,10-tetrahydro-2-carboxymethyl-14-sulfonato-16,16,18,18-tetramethyl-7aH-bisindolinium[3,2-a,3',2'-a]pyrano[3,2-c,5,6-c]dipyridin-5-ium NHS ester (1.0mg) was added N-(2-aminoethyl)-3-oxoandrost-4-ene-17-carboxamide (0.6mg), diisopropylethylamine (0.01ml) and dichloromethane (0.2ml). This mixture was placed on a roller for 18 hours and then purified by preparatory HPLC [column: Phenomenex Jupiter 10u C18 300A 250x21.2mm. Method: 20ml/min, 5% to 50% B over 30min (A=water 0.1% TFA, B=CH3CN 0.1% TFA). Peaks were detected at 559nm. RT (product) ~27min]. Relevant fractions were combined and concentrated on a rotary evaporator. The material was then freeze dried to give 1.0mg of the desired product (Formula XIV). Mass spectrum : 902 (M+H)

20

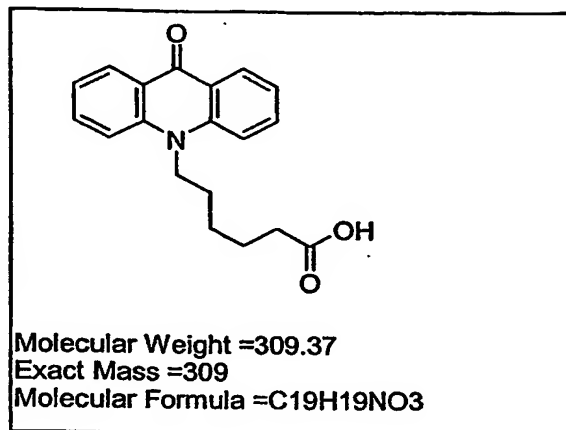
iv) Ethyl 6-(9-oxoacridin-10(9H)-yl)hexanoate

Formula (XV)

5

To 9(10H)-acridone (1.0g) was added tetrahydrofuran (15ml) under an atmosphere of nitrogen. Sodium hydride (0.25g) was added with stirring; after 30minutes ethyl 6-bromohexanoate (1.12ml) was added and the mixture heated to reflux for 18 hours. After this time water (10ml) was added and the layers separated. The organic layer was dried over magnesium sulfate, filtered and evaporated to dryness. Dry flash column chromatography was performed to give 0.85g of the required material (Formula XV). Mass spectrum : 338 (M+H).

15

v) 6-(9-oxoacridin-10(9H)-yl)hexanoic acid

5

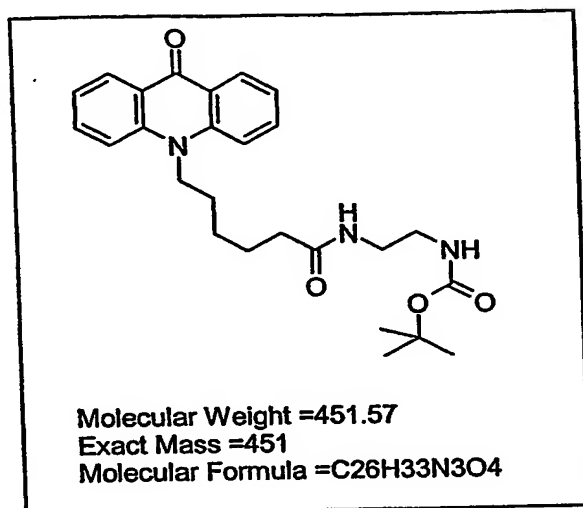
## Formula (XVI)

To ethyl 6-(9-oxoacridin-10(9H)-yl)hexanoate (0.80g) was added acetic acid (9ml) and 2M hydrochloric acid (2.5ml). The mixture was heated to 100°C for 18 hours after which time the volatile components were removed on a rotary evaporator. Diethyl ether was added (25ml) and the mixture stirred for 15 minutes. The resulting material was filtered off and air dried to give 0.36g final product (Formula XVI). Mass spectrum : 310 (M+H).

15



vi) Tert-Butyl 2-([6-(9-oxoacridin-10(9H)-yl)hexanoyl]amino)ethylcarbamate



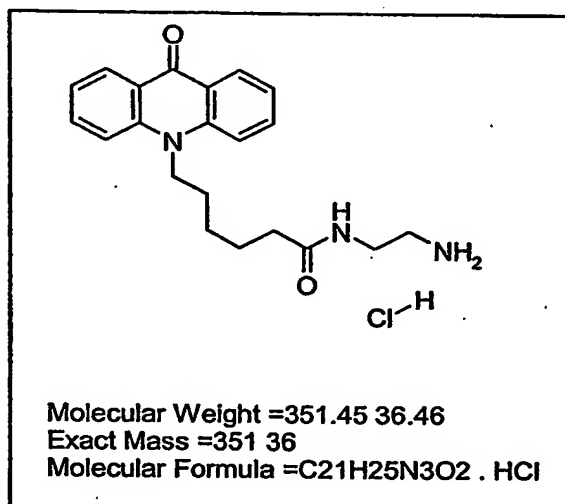
5

Formula (XVII)

To 0.48g of 6-(9-oxoacridin-10(9H)-yl)hexanoic acid was added  
10 dichloromethane (6ml) and thionyl chloride (0.2ml). This mixture was heated  
to reflux for 1 hour after which time the volatile components were removed by  
application of vacuum. To the resulting oil was added dichloromethane (3ml),  
pyridine (3ml) and t-butyl N-(2-aminoethyl)carbamate (250mg). This mixture  
was stirred for 18 hours after which time it was poured into 0.5M sodium  
15 hydroxide solution (15ml) and extracted with dichloromethane (2x10ml). The  
combined dichloromethane solutions were washed with 0.1M hydrochloric  
acid solution, dried over magnesium sulfate, filtered and evaporated to dryness.  
The resulting material was purified by column chromatography to give 0.30g  
of a solid final product (Formula XVII).

20

vii) N-(2-aminoethyl)-6-(9-oxoacridin-10(9H)-yl)hexanamide hydrochloride

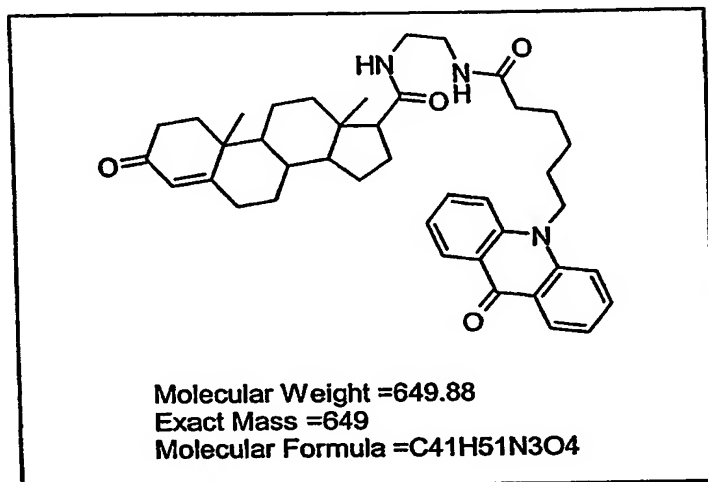


5

## Formula XVIII

To 0.30g of tert-butyl 2-[[6-(9-oxoacridin-10(9H)-yl)hexanoyl]amino]ethylcarbamate was added dichloromethane (DCM; 30ml). HCl (g) was bubbled through the solution for 10 minutes. After this time  
10 the mixture was filtered and washed with DCM (3x20ml) to give the desired product (0.15g; Formula XVIII). Mass spectrum : 352 (M+H).

viii) 3-oxo-N-(2-([6-(9-oxoacridin-10(9H)-yl)]hexanoyl]amino)ethyl)androst-4-ene-17-carboxamide



Formula XIX

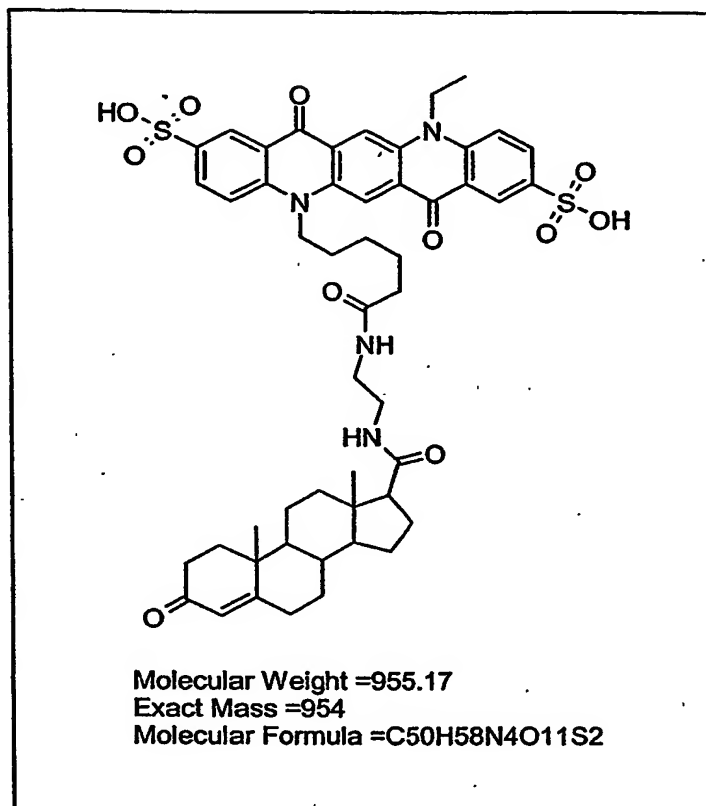
5

To 0.082g of 4-androsten-3-one-17B carboxylic acid was added DMF (5ml), DIPEA (0.045ml) and O-(N-Succinimidyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (0.08g). After stirring for 1 hour N-(2-aminoethyl)-6-(9-oxoacridin-10(9H)-yl)hexanamide hydrochloride (compound XVII) (0.10g) was added and stirring continued for 3 days. Preparatory HPLC was performed [column: Phenomenex Jupiter 10u C18 300A 250x21.2mm; 20ml/min, 5% to 95% B over 30min (A=water 0.1% TFA, B=CH<sub>3</sub>CN 0.1% TFA). Peaks were detected at 280nm. RT (product) ~23min] and the relevant fractions combined and concentrated on a rotary evaporator. Freeze drying gave 0.0683g of product (Formula XIX). Mass spectrum : 650 (M+H).

15

ix) 5-ethyl-7,14-dioxo-12-{6-oxo-6-[(2-[(3-oxoandrost-4-en-17-yl)carbonyl]amino}ethyl)amino]hexyl}-5,7,12,14-tetrahydroquino[2,3-b]acridin-2,9-disulfonic acid

5



Formula XX

- 10 N-(2-aminoethyl)-3-oxoandrost-4-ene-17-carboxamide (1.0mg) was dissolved in dichloromethane (1ml) and a solution of 5-{6-[(2,5-dioxopyrrolidin-1-yl)oxy]-6-oxohexyl}-12-ethyl-7,14-dioxo-5,7,12,14-tetrahydroquino[2,3-b]acridin-2,9-disulfonic acid (2mg) in DMF (1ml) added. DIPEA (0.02ml) was added and the mixture stirred at room temperature for 1 hour. After this time preparatory
- 15 HPLC was performed to give 1.6 mg of the desired material (Formula XX).  
Mass spectrum: 956 (M+H).

#### Aromatase Assay

- 20 NADPH was prepared to a final concentration of 1mM in 100mM disodium hydrogen phosphate buffer pH7.4. The labelled substrate (i.e. 3-oxo-N-(2-[[6-

(9-oxoacridin-10(9H)-yl)hexanoyl[amino}ethyl)androst-4-ene-17-carboxamide; Formula XIX) or chromophore alone (6-(9-oxoacridin-10(9H)-yl)hexanoic acid – compound XVI) was added to the solution of NADPH to a final concentration of 2µM.

5

100µl of this reagent was dispensed in to replicate wells of a 96 well microtitre plate. To each well was dispensed 20µl-30µl of either the CYP19 (aromatase) containing microsomes or control microsomes (no CYP19). All microsomes were adjusted to the same protein concentration with assay buffer. The plates were incubated at 37°C for 1 hour and then fluorescence intensity measurements were recorded on the Envision Plate Reader (Perkin Elmer, US), excitation 405nm/emission BFP450nm.

10

Figure 2 compares the fluorescence intensity data from a 'buffer only' treatment and microsomes with and without aromatase activity. As can be seen, microsomes containing active aromatase produce a greater decrease in fluorescence intensity compared to the corresponding control microsome preparation. The decrease in signal in the presence of microsomes may represent quenching of the substrate signal due to the presence of protein/lipid.

20

The fluorescence signal was seen to be proportional to the amount of enzyme/microsome present in the assay. Figure 3 depicts the effect of microsome volume on the assay signal. A microsome volume of 20µl generated a 17.5% decrease in intensity relative to the control reaction. This was further increased to 24.2% in the presence of 30µl of microsome preparation.

25

Figure 4 illustrates the NADPH dependence of aromatase (CYP19 in the diagram) activity. A 24% decrease in fluorescence was observed in the presence of additional NADPH. This compared to 16% in the absence of additional co-factor. The signal observed in the absence of additional NADPH may reflect the presence of NAD(P)H in the enzyme preparation.

30

Figure 5 shows the specificity of the enzyme for its substrate. In the presence of 20µl of CYP19 aromatase preparation a 17.5% change in intensity relative to the control was observed for the labelled steroid reporter. The chromophore alone (i.e. 6-(9-oxoacridin-10(9H)-yl)hexanoic acid – compound

- 5 XVI) generated a 5% change in intensity when incubated with CYP19 microsomes. Therefore, the observed decrease in fluorescence intensity was not due to the enzyme acting directly on the chromophore.

1. A compound of Formula 1:



(I)

wherein

R is a fluorescent dye molecule;

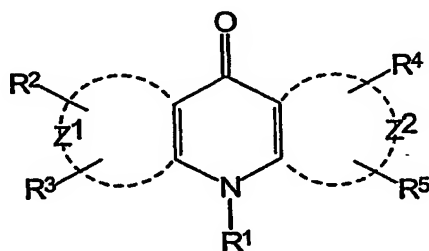
L is an optional linkage group containing one or more atoms comprising hydrocarbon chains which may also contain other atoms such as N, O and S; and

S is molecule comprising a substrate group of the enzyme aromatase

characterised in that the fluorescence signal of said compound changes in respect of fluorescence intensity or fluorescence lifetime when the compound is acted upon by an enzyme with aromatase activity.

2. A compound according to claim 1 wherein R is selected from the group consisting of fluorescein, rhodamine, coumarin, BODIPY<sup>TM</sup> dye, phenoxazine, cyanine, Alexa<sup>TM</sup> fluors, merocyanine, Cy3B, Cy5, Cy5.5, Cy7, acridone, quinacridone and squarate dyes

3. A compound according to claim 1 or 2 wherein said R is an acridone dye of Formula II:



(II)

wherein:

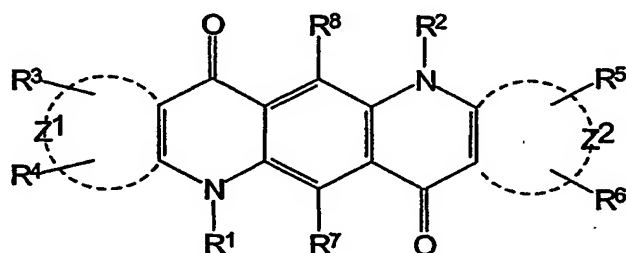
groups R<sup>2</sup> and R<sup>3</sup> are attached to the Z<sup>1</sup> ring structure and groups R<sup>4</sup> and R<sup>5</sup> are attached to the Z<sup>2</sup> ring structure;

Z<sup>1</sup> and Z<sup>2</sup> independently represent the atoms necessary to complete one or two fused ring aromatic or heteroaromatic systems, each ring having five or

six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

$R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$  and  $R^5$  are independently selected from hydrogen, halogen, amide, hydroxyl, cyano, amino, mono- or di- $C_1$ - $C_4$  alkyl-substituted amino, sulphhydryl, carbonyl,  $C_1$ - $C_6$  alkoxy, aryl, heteroaryl,  $C_1$ - $C_{20}$  alkyl, aralkyl; the group -E-F where E is a spacer group having a chain from 1-60 atoms selected from the group consisting of carbon, nitrogen, oxygen, sulphur and phosphorus atoms and F is a target bonding group; and the group  $-(CH_2)_nY$  where Y is selected from sulphonate, sulphate, phosphonate, phosphate, quaternary ammonium and carboxyl and n is zero or an integer from 1 to 6.

4. A compound according to claim 1 or 2 wherein R is a quinacridone dye of Formula III:



(III)

wherein:

groups  $R^3$  and  $R^4$  are attached to the  $Z^1$  ring structure and groups  $R^5$  and  $R^6$  are attached to the  $Z^2$  ring structure;

$Z^1$  and  $Z^2$  independently represent the atoms necessary to complete one or two fused ring aromatic or heteroaromatic systems, each ring having five or six atoms selected from carbon atoms and optionally no more than two atoms selected from oxygen, nitrogen and sulphur;

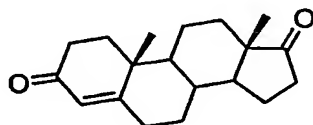
$R^1$ ,  $R^2$ ,  $R^3$ ,  $R^4$ ,  $R^5$ ,  $R^6$ ,  $R^7$  and  $R^8$  are independently selected from hydrogen, halogen, amide, hydroxyl, cyano, amino, mono- or di- $C_1$ - $C_4$  alkyl-substituted amino, sulphhydryl, carbonyl, carboxyl,  $C_1$ - $C_6$  alkoxy, aryl, heteroaryl,  $C_1$ - $C_{20}$  alkyl, aralkyl; the group -E-F where E is a spacer group having a chain from 1-60 atoms selected from the group consisting of carbon, nitrogen, oxygen, sulphur and phosphorus atoms and F is a target bonding group; and the group  $-(CH_2)_nY$  where Y is selected from sulphonate, sulphate, phosphonate,



phosphate, quaternary ammonium and carboxyl and n is zero or an integer from 1 to 6.

5. A compound according to any of claims 1 to 4 wherein L is a linker group containing from 1 to 40 linked atoms selected from carbon atoms which may optionally include one or more groups selected from  $\text{-NR'}$ -,  $\text{-O-}$ -,  $\text{-S-}$ -,  $\text{-CH=CH-}$ -,  $\text{-C}\equiv\text{C-}$ -,  $\text{-CONH-}$  and phenylenyl groups, wherein R' is selected from hydrogen and C1 to C4 alkyl.
6. A compound according to any of claims 1 to 5, wherein L is a linker group containing from 2 to 30 atoms.
7. A compound according to any of claim 1 to 6, wherein L is a linker group containing from 6 to 20 atoms.
8. A compound according to any of claims 1 to 7, wherein L is a linker group selected from the group:  $\{(-\text{CHR}')_p\text{-Q-}(-\text{CHR}')_r\}_s$
- where each Q is selected from  $\text{CHR'}$ ,  $\text{NR'}$ , O,  $\text{-CH=CH-}$ , Ar and  $\text{-CONH-}$ ;
- each R' is independently hydrogen or C1 to C4 alkyl;
- each p is independently 0 to 5;
- each r is independently 0 to 5;
- and s is either 1 or 2.
9. A compound according to claim 8, wherein Q is selected from the group consisting of  $\text{-CHR'}$ -,  $\text{-O-}$  and  $\text{-CONH-}$ , where R' is hydrogen or C1 to C4 alkyl.

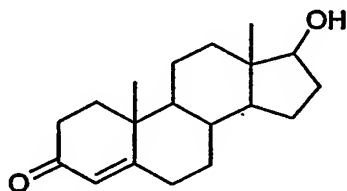
10. A compound according to any of claims 1 to 9 wherein S is androstenedione of Formula IX or a derivative thereof.



5

(IX)

11. A compound according to any of claims 1 to 9 wherein S is testosterone of Formula X or a derivative thereof.

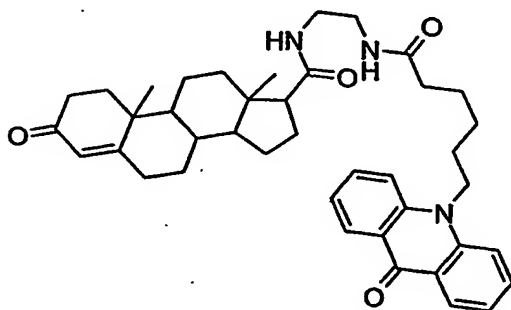


10

(X)

12. A compound according to any preceding claim of Formula XIX

15



20

(XIX)

13. A method for measuring aromatase activity in a sample, the method comprising the steps of:

- 5           i)     measuring the fluorescence intensity or fluorescence lifetime of a compound according to any preceding claim prior to adding it to said sample;
- ii)     adding said compound to said sample under conditions which favour aromatase activity, and
- 10          iii)    measuring a change in fluorescence intensity or fluorescence lifetime of said compound following step ii);

wherein said change in fluorescence intensity or fluorescence lifetime can be used to determine aromatase activity.

- 15   14. A method according to claim 13 wherein the sample is selected from the group consisting of extract, cell, tissue and organism.

15. A method of screening for a test agent whose effect upon the activity of aromatase is to be determined, said method comprising the steps of:

20

- i)     performing the method of claim 13 or 14 in the presence of said agent; and
- ii)    comparing the activity of said aromatase in the presence of the agent with a known value for the activity of aromatase in the absence of the agent;

25

wherein a difference between the activity of the aromatase in the presence of the agent and said known value in the absence of the agent is indicative of the effect of the test agent upon the activity of aromatase.

- 30   16. The method according to claim 15, wherein the known value is stored upon an electronic database.

17. A method of screening for a test agent whose effect upon the activity of aromatase is to be determined, said method comprising the steps of:

- i) performing the method of claim 15 or 16 in the presence and in the absence of the agent; and
- ii) determining the activity of said enzyme in the presence and in the absence of the agent;

wherein a difference between the activity of aromatase in the presence and in the absence of the agent is indicative of the effect of the test agent upon the activity of aromatase.

18. The method according to claim 17 wherein said difference in activity between the activity of aromatase in the absence and in the presence of the agent is normalised, stored electronically and compared with a value of a reference compound.

19. A method for measuring cellular distribution of the compound of any of claims 1 to 12, wherein the compound is capable of being taken up by a living cell, the method comprising the steps of:

- i) measuring the fluorescence intensity or fluorescence lifetime of the compound in a cell-free environment or a parental host cell;
- ii) adding the compound to one or more cells or a cell engineered to over-express aromatase, and
- iii) measuring the fluorescence intensity or lifetime of the compound following step ii);

wherein a change in fluorescence intensity or fluorescence lifetime indicates aromatase activity and can be used to determine the distribution of the compound.

20. The method of claim 19, wherein said cell is selected from the group consisting of mammalian, plant, insect, fish, avian, bacterial and fungal cells.

21. Use of a compound according to any of claims 1 to 12 for measuring aromatase activity as an *in vitro* or an *in vivo* imaging probe.

22. Kit comprising:

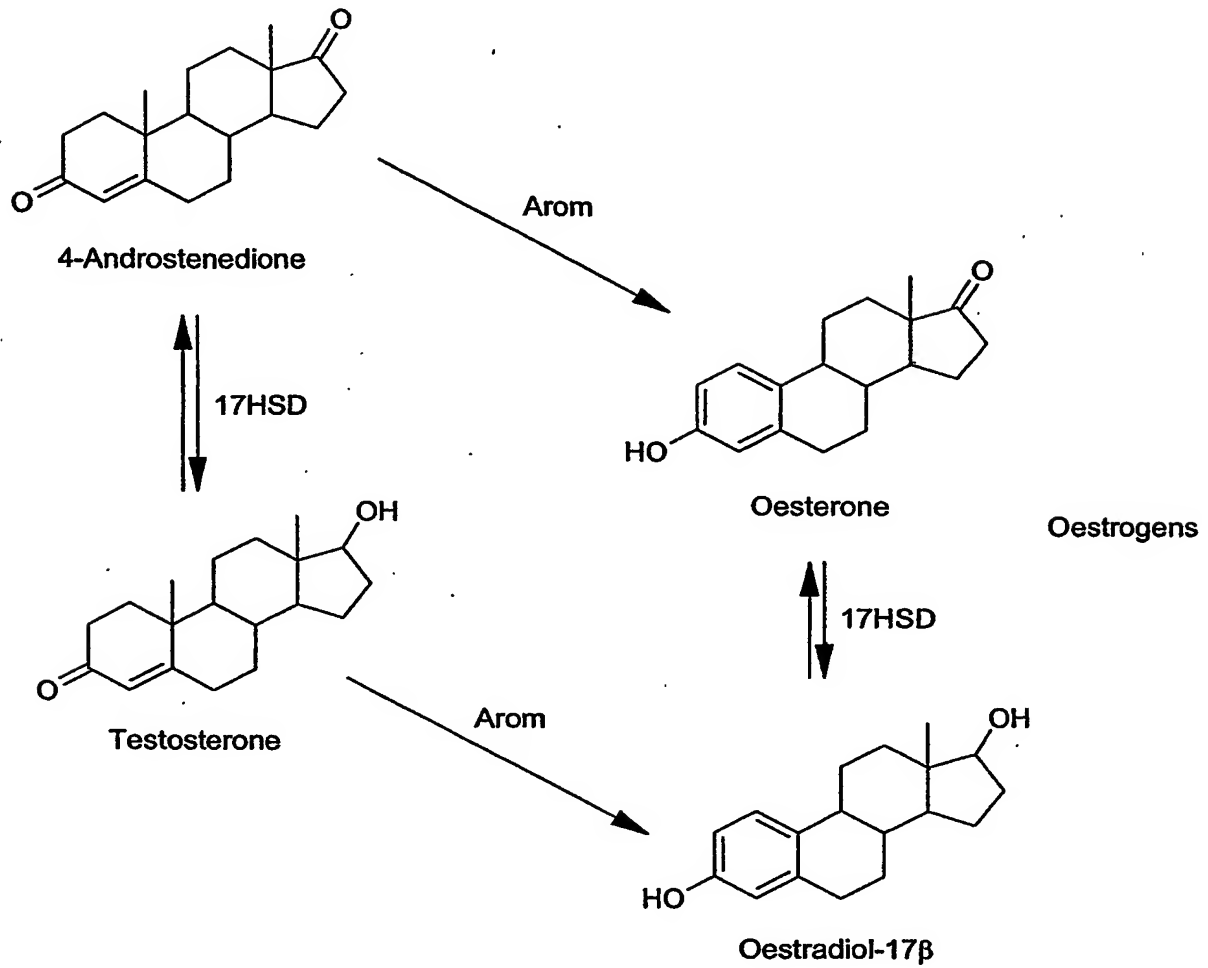
- i) a compound according to any of claims 1 to 12;
- ii) an assay buffer; and optionally
- iii) a stop buffer.

**Abstract**

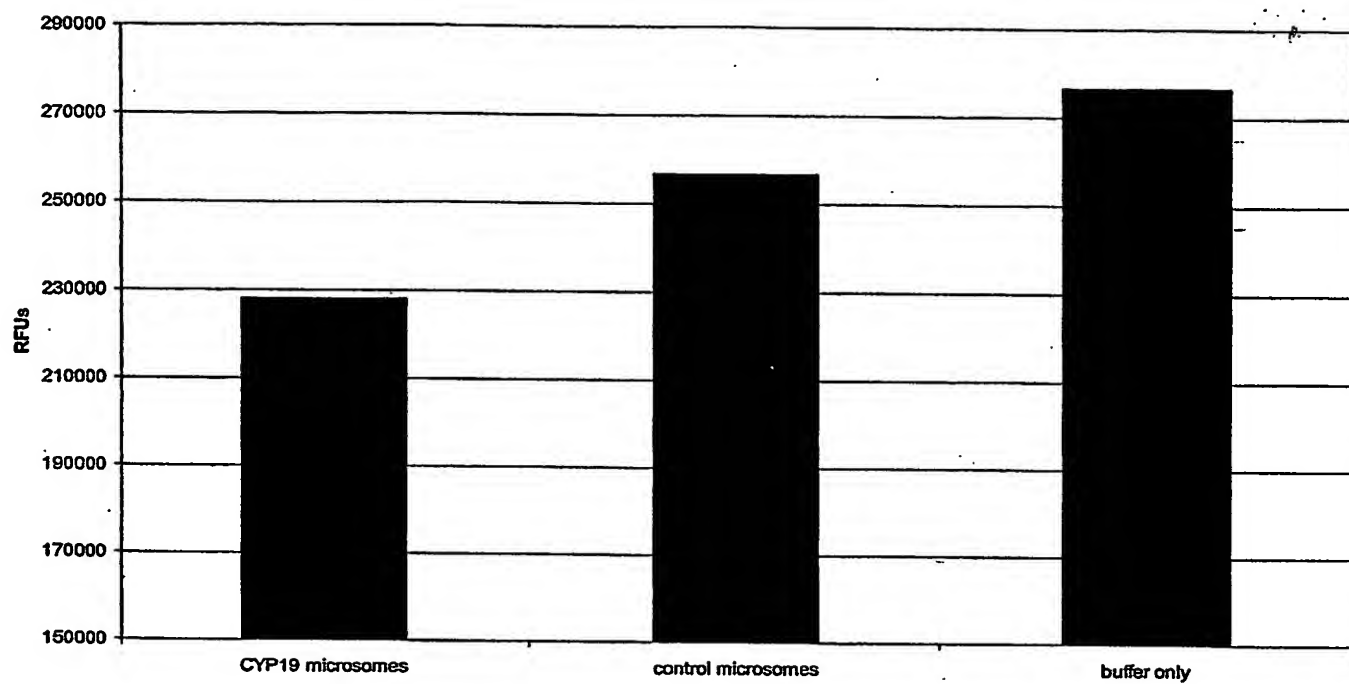
The present invention relates to compounds useful for measuring aromatase activity. The invention further provides methods for measuring aromatase activity and for screening test agents which modulate aromatase activity. A kit  
5 is also provided for use in such screening methods.

10

15

**Figure 1 : Conversion of 4-Androstenedione to Oestrone by Aromatase**

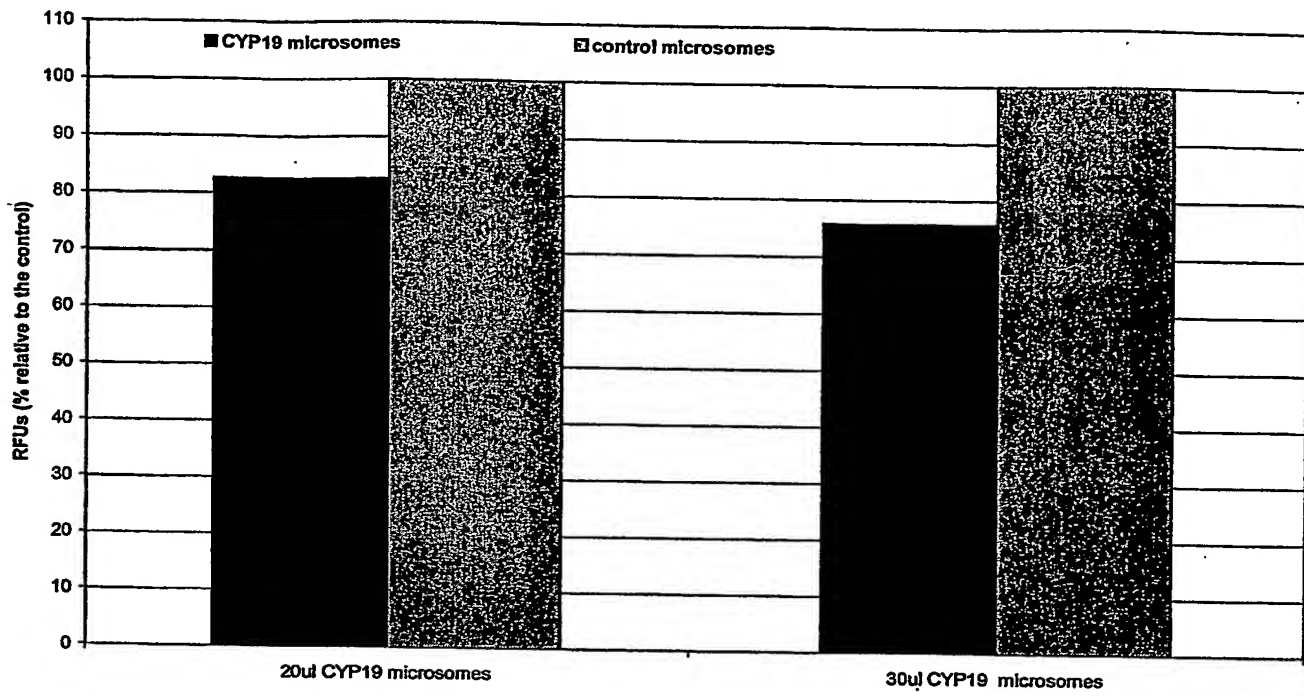
## Effect of assay conditions on signal



**Figure 2.** Comparison of buffer only, control and CYP19 microsomes on assay signal.

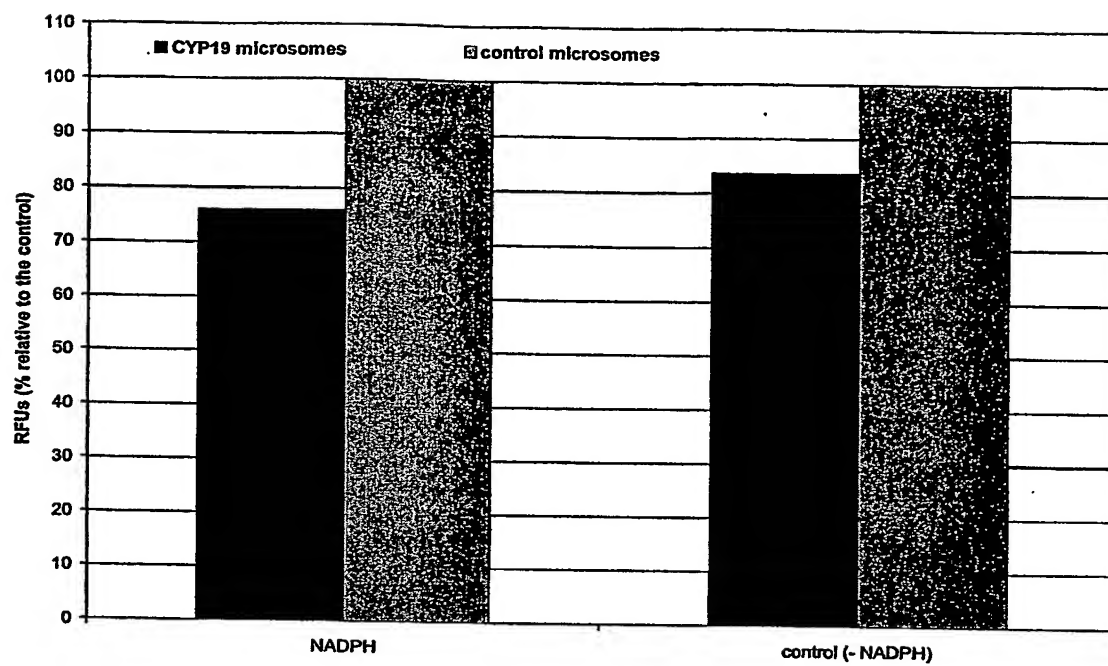


## Effect of microsome volume on assay signal



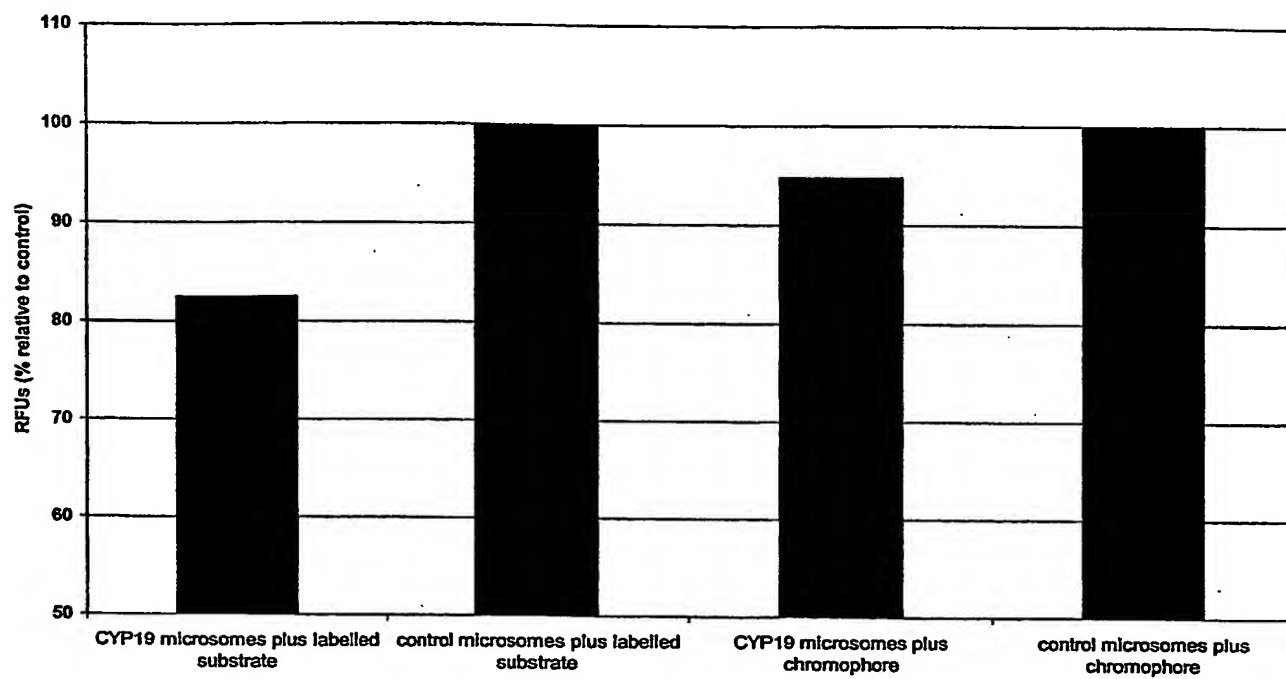
**Figure 3.** Effect of microsome volume on assay signal.

## Effect of NADPH on assay signal



**Figure 4.** NADPH dependence of CYP19.

Comparison of labelled aromatase substrate and chromophore

**Figure 5. Specificity of the aromatase substrate.**

**PCT/GB2004/003341**



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**